A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins

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Background: All organisms perceive and respond to a profusion of environmental and endogenous signals that influence growth, development and behaviour. The G-protein signalling pathway is a highly conserved mechanism for transducing extracellular signals, and the superfamily of receptors that have seven transmembrane (7TM) domains is a primary element of this pathway. Evidence that heterotrimeric G proteins are involved in signal transduction in plants is accumulating, prompting speculation that plant 7TM receptors might exist.

Results: Using information in the dbEST database of expressed sequence tags, we isolated an *Arabidopsis thaliana* gene (*GCR1*) that encodes a protein with seven predicted membrane-spanning domains and other features characteristic of 7TM receptors. The protein shows 18–23% amino-acid identity (46–53% similarity) to, and good colinear alignment with, 7TM receptors from three different families. Its highest sequence identity is with the *Dictyostelium* cAMP receptors. *GCR1* is expressed at very low levels in the roots, stems and leaves of *Arabidopsis*; it is a single-copy gene which maps close to the restriction fragment length polymorphism marker m291 on chromosome 5. Transgenic *Arabidopsis* expressing antisense *GCR1* under the control of the constitutive cauliflower mosaic virus *35S* promoter have reduced sensitivity to cytokinins in roots and shoots, yet respond normally to all other plant hormones. This suggests a functional role for *GCR1* in cytokinin signal transduction.

Conclusions: *GCR1* encodes the first 7TM receptor homologue identified in higher plants and is involved in cytokinin signal transduction. This discovery suggests that 7TM receptors are ancient and predate the divergence of plants and animals.

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Background

One of the most highly conserved mechanisms for transducing extracellular signals is the G-protein signalling pathway [1]. There is increasing evidence from molecular, biochemical and cell biological studies that higher plants employ G-protein signalling pathways [2]. High-affinity [α^{32}P]GTP-binding and [^{35}S]GTP-γ-S-binding activity, along with polypeptides that can be ADP-ribosylated or will crossreact with antisera against Gα subunits or Gα-subunit peptides, has been detected in microsomal and plasma membranes from a number of plant species [2]. Genomic and cDNA clones encoding Gα subunits have been isolated from *Arabidopsis thaliana* (*GPA1*), tomato (*TGA1*) [2], *Lotus japonicus* (*LjGPA1*) [3], soybean (*SGA1*) [4] and rice (*RGA1*) [5,6]. The predicted protein products have 74–87% amino-acid sequence identity to one another and 34–42% identity to non-plant Gα subunits. It is likely that these clones represent a single class of plant Gα subunit. Although Gβ subunit cDNAs have been cloned from maize and *Arabidopsis* [7], a Gγ subunit has yet to be identified in plants. It is possible, though not proven, that plant Gα and Gβ subunits are functional homologues of their mammalian and non-plant counterparts.

A wealth of evidence supports a role for G proteins in the regulation of K^+ influx channels of stomatal guard cells [8]. The GTP analogues GTP-γ-S and GDP-β-S, cholera and pertussis toxins, and the G-protein agonist Mas 7, have been shown to modulate guard cell inward K^+ channel activity in a complex manner that may indicate the involvement of multiple G-protein signalling pathways in the regulation of stomatal aperture [9–14]. Similar studies have implicated G proteins in responses to plant hormones [2], blue and red light [2,15,16], fungal elicitors [2] and pathogen resistance and pathogen-related gene expression [17]. These observations suggest that seven transmembrane (7TM) receptors might exist in plants but, although there has been speculation as to their existence [13,18], no candidate plant 7TM receptors have been found.

To test the hypothesis that 7TM receptors might exist in plants, we identified, in the dbEST database of expressed

sequence tags (EST) [19], seven *Arabidopsis*, one rice and one pine EST whose predicted products have similarities to 7TM receptors. Using amplimers based on one of these sequences, we isolated a gene, *GCR1*, from *Arabidopsis* that encodes a protein with seven predicted transmembrane domains and significant colinear similarity to 7TM receptors. The GCR1 protein is most closely related to the *Dictyostelium* cAMP receptors. *GCR1* is a single-copy gene on chromosome 5 near to the restriction fragment length polymorphism (RFLP) marker m291 (76.4 cM) and is expressed at very low levels in roots, stems and leaves of seedlings and adult plants. To explore the possible function of *GCR1*, we transformed *Arabidopsis* with antisense cDNA under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. In the second generation (T2), six out of 24 antisense lines produced segregants with a distinct phenotype that involved reduced cotyledon and leaf expansion and production of a single flowering stem at the seven-leaf stage. This phenotype is similar, though not identical, to that of the cytokinin-resistant *Arabidopsis* mutant *cyr1*, which maps to the same region of chromosome 5. The phenotype was stably inherited into the T3 generation of a selected transgenic *Arabidopsis* line containing the antisense *GCR1* construct, and we demonstrated that seedlings of this line have reduced sensitivity to the cytokinin benzyladenine in roots and shoots and that responses to all other plant hormones were unaffected. We sequenced *GCR1* from homozygous *cyr1* and *CYR1* seedling DNA and found no differences in the deduced amino-acid sequence of the open reading frame (ORF) or at the intron/exon splice sites, indicating that *GCR1* is not allelic to the *cyr1* mutation. *GCR1* mRNA levels in homozygous *cyr1* mutants were similar to those in the wild-type plant. Our data suggest that higher plants possess a 7TM receptor homologue that is involved in cytokinin signalling.

Results and discussion

Candidate 7TM receptor homologues in the dbEST

Searches of the dbEST identified seven *Arabidopsis*, one rice and one pine EST whose predicted products have high similarity to 7TM receptors (Table 1). We concentrated on ATTS2866 (330 bp) which showed highest similarity to the *Dictyostelium discoideum* cAMP receptor CAR1 [20]. The other end of the clone (ATTS1113) was not similar to 7TM receptors. A search of the nucleotide sequence databases with the ATTS2866 sequence using the BLASTN algorithm found a homologous EST (dbEST ID 376) with no similarity to 7TM receptors. Using nested primers in a PCR, we found that the sequence of the EST (dbEST ID 376) overlapped, and lay 5′ to, ATTS2866. Using primers based on the 5′ end of the EST (dbEST ID 376) and the 3′ end of ATTS1113, we generated the genomic clone *GCR1* (GenBank accession number U95142; see Supplementary material) and a 1281 bp cDNA. To confirm that *GCR1* was not unique to *Arabidopsis*, degenerate primers based on the ORF were used to isolate a homologue from *Brassica napus* (GenBank accession number U95144; see Supplementary material). Exons of the two genes are 91% identical with 100% amino-acid conservation over the region examined. The similarity in intron/exon organization (Figure 1a) suggests structural and functional conservation.

We prepared a Marathon rapid amplification of cDNA ends (RACE) library (see Materials and methods) from 7 day old etiolated *Arabidopsis* seedlings. Six 5′ and four 3′ RACE clones were isolated and sequenced. The consensus *GCR1* cDNA (GenBank accession number U95143; Figure 1b) has a single long ORF of 326 amino acids. Two in-frame stop codons upstream of the postulated initiating methionine confirm that the cDNA is full length. Heterogeneity in the length of the 3′-untranslated region recovered in different clones (Figure 1b) suggested multiple polyadenylation sites.

GCR1 encodes a 7TM receptor homologue

Hydropathy analysis [21] of the *GCR1* ORF revealed a hydrophobic amino terminus, seven hydrophobic regions of 19–25 amino acids separated by hydrophilic domains, and a hydrophilic carboxyl terminus (Figure 2a). Analysis of the predicted protein sequence of GCR1 using the PHDhtp algorithm [22], and against the TMbase database using the TMpred algorithm [23], suggested that the amino terminus is extracellular, that there are seven

Table 1

ESTs that have sequence similarity to 7TM receptors.

dbEST ID	Protein match	*p* value
ATTS2866	cAMP receptor CAR1 (pir A41238)	2.4e-13
ATTS1764	G-protein-coupled receptor homologue (sp P32229)	2.79e-04
ATTS551531	Dopamine receptor D4 (pir S15079)	9.1e-03
RICS10503A	Mas-related G-protein-coupled receptor (pir A39485)	4.59e-03
377104	Melatonin receptor (sp P48039)	4.4e-03
ATTS20443	cAMP receptor CAR4 (pir A54813)	7.3e-02
ATTS551725	cAMP receptor CAR4 (pir A54813)	5.9e-02
ATTS551563	β-3-Adrenergic receptor (pir S33751)	4.4e-02
ATTS471578	α-1-Adrenergic receptor (pir A40491)	1.5e-02

Free text and protein sequence queries were made at http://www2.ncbi.nlm.nih.gov/dbST/dbest_query.html and http://lenti.med.umn.edu/gst/MotifExplorer.html. EST sequences were then compared with non-redundant protein sequence databases using the Basic Local Alignment Search Tool (BLASTX algorithm) to identify those that showed significant similarity to known 7TM receptors. The table lists ESTs which have similarity to 7TM receptors that extends across a minimum of two transmembrane regions. The respective Protein Information Resource (pir) and Swissprot (sp) accession numbers are indicated after the names of the proteins.

transmembrane domains connected by alternating intracellular and extracellular loops, and that the carboxyl terminus is intracellular. The distribution of charged amino acids is consistent with this proposed structure, with 24 of the 31 lysine or arginine residues predicted to be intracellular. The proposed structure of GCR1 is highly characteristic of 7TM receptors [1] (Figure 2b).

GCR1 has a number of other features that are common to many 7TM receptors (Figure 2b). The majority of 7TM receptors contain cysteines in the second and third extracellular loops that are thought to form a disulfide bridge. Mutation of these is known to affect ligand binding and receptor expression [1]. GCR1 also has cysteines in the first and second extracellular loops that could form a disulfide bridge. The second extracellular loop of GCR1 has a potential *N*-linked glycosylation site and this is consistent with many 7TM receptors which have potential glycosylation sites in either or both of the amino-terminal extracellular region and second extracellular loop. The predicted second transmembrane domain (TM2) of GCR1 contains the motif LAXXD (in the single-letter amino-acid code, X can be any amino acid) which is highly conserved in the family A of 7TM receptors ([24]; see below). The aspartate of this motif is one of the most highly conserved amino acids in 7TM receptors and is thought to be involved in transduction of agonist binding [1]. At the boundary between the third transmembrane domain (TM3) and the second cytoplasmic loop, GCR1 has an arginine that is conserved in virtually all 7TM receptors [24], preceded by a highly conserved histidine. Other conserved residues include tryptophan in the fourth transmembrane domain (TM4), the motif FXXPXXXXXXY in the fifth transmembrane domain (TM5) and asparagine and tyrosine in the seventh transmembrane domain (TM7) [1,24,25]. In addition, GCR1 has multiple serines and a threonine in the carboxyl terminus that may be sites of reversible phosphorylation and regulation by receptor kinases [1]; for example, Ser303 in the *Dictyostelium* cAMP receptor CAR1 is phosphorylated during cAMP-binding [26], and the equivalent residue, Ser319 in GCR1, is conserved.

The superfamily of 7TM receptors has been classified into six families on the basis of sequence similarity in the transmembrane domains and receptors in each of these families are thought to share a common ancestor [24]. In general, these similarities are greatest for species homologues (85–90% identity), and sub-types of the same receptor (60–80% identity). Members of the same family can show as little as 35–40% identity, and unrelated 7TM receptors

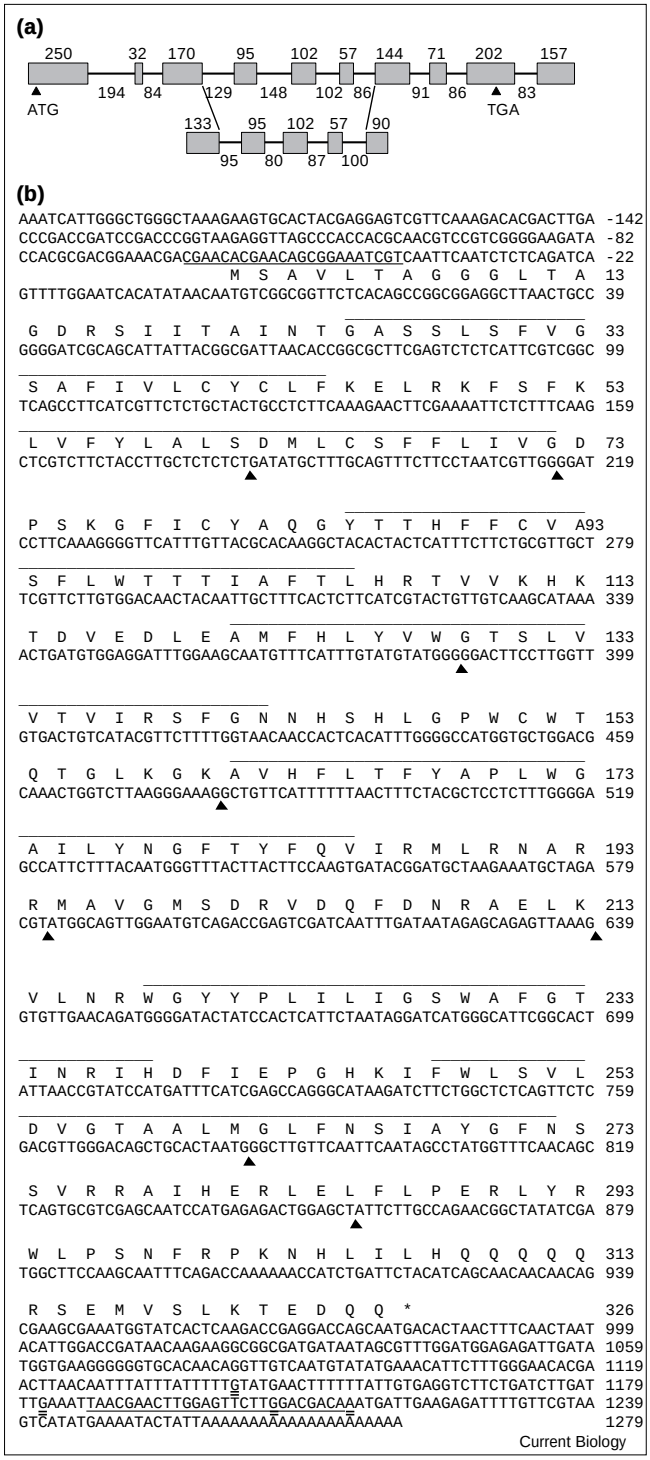
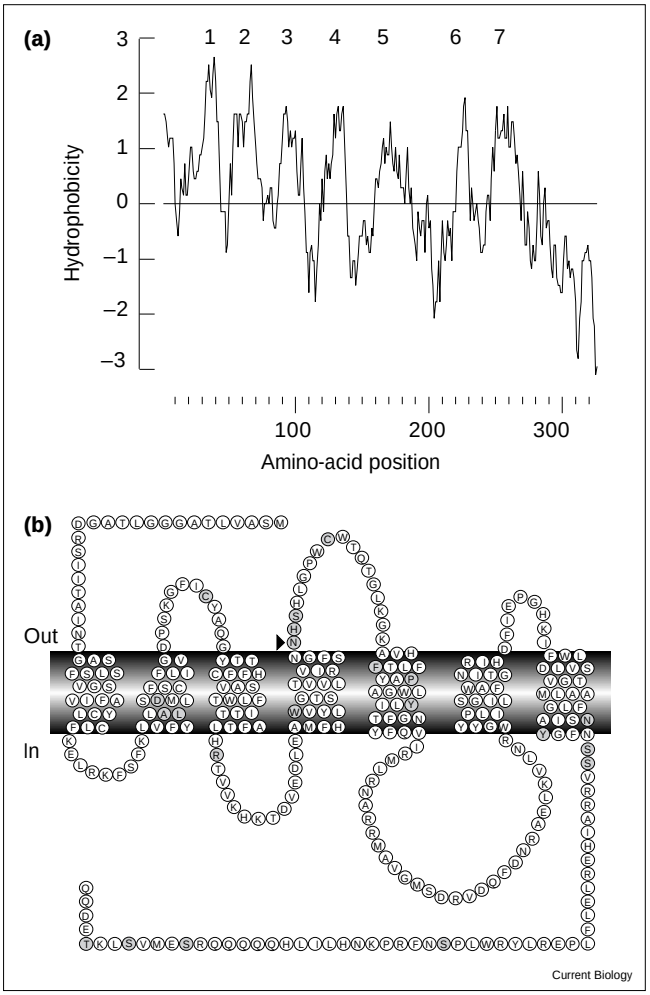


Figure 1

Sequence of *GCR1*. (a) Intron/exon organisation and relationship between the *GCR1* gene of *A. thaliana* (upper) and *B. napus* (lower); the 5′ and 3′ ends of *B. napus GCR1* have not yet been cloned. Numbers above exons (grey boxes) and below introns (lines) indicate their length in nucleotides. The positions of the start (ATG) and stop (TGA) codons in the *A. thaliana* sequence are indicated. (b) Nucleotide sequence of the *A. thaliana GCR1* cDNA and the deduced amino-acid sequence (in single-letter amino-acid code). The seven potential membrane-spanning domains are indicated by solid lines above the amino-acid sequence, and the positions of introns within the coding region are indicated by triangles. The DNA sequences used for primer design are underlined, and alternative polyadenylation sites are double underlined.

may be only 20–25% identical in the transmembrane domains [1]. To determine which 7TM receptors GCR1 is most similar to, we compared the amino-acid sequence of GCR1 against protein sequence databases using the FASTA and BLAST algorithms, and against the G-protein-coupled receptor database [24] using FASTA. GCR1 showed highest similarity to the four members of family E, the *Dictyostelium* cAMP receptors (CAR1–4). Similarity with CAR1–4 ranged from 20% to 23% identity over a region of between 210 and 285 amino acids that encompassed all 7TM regions. There was significant similarity between GCR1 and the family B receptors for corticotrophin releasing factor and calcitonin and a number of serotonin and olfactory receptors of family A. Table 2 compares GCR1 with the highest scoring sequences from these families. Similarities between GCR1 and the serotonin receptors were greatest over a 49 amino-acid region encompassing TM1, the first intracellular loop and part of TM2 in which there was 34% amino-acid identity (61% similarity).

Figure 2



GCR1 encodes a polypeptide with seven potential membrane-spanning domains. (a) Kyte–Doolittle hydrophilicity plot. Analysis of the amino-acid sequence of GCR1 was performed using the Peptide Structure program of the University of Wisconsin Genetics Computer Group (GCG) package version 8.0 using a window of integration for hydrophilicity measurements set at 10, and results are plotted as a hydrophobicity index. A similar result was obtained using a window of integration for hydrophilicity measurements set at 7. Predicted transmembrane helices are numbered 1–7. (b) Serpentine model of GCR1. Residues conserved in 7TM receptors are shown on a grey background. The potential *N*-linked glycosylation site is indicated (arrowhead).

GCR1 was optimally aligned against CAR1 using the PileUp algorithm of the GCG package version 8.0 (Figure 3a). This analysis showed that sequence similarity between these proteins extended across the whole of GCR1, and that the proteins had a similar arrangement of intracellular and extracellular loops and transmembrane domains. The alignment introduced gaps into some of the transmembrane domains, particularly TM5, 6 and 7, suggesting that the proteins may be less closely related over these regions. In addition, the carboxy-terminal domain of GCR1 is shorter than the corresponding region of CAR1. Phylogenetic analysis (Figure 3b) indicated that GCR1 is most closely related to the *Dictyostelium* cAMP receptors (CAR1–4) and suggested that it may form the basis of a new family of 7TM receptors.

Expression and genomic map location of *GCR1* in *Arabidopsis*

Northern blot analysis of polyA$^+$ RNA isolated from 7 day old etiolated seedlings revealed very low levels of a transcript of the expected size (Figure 4a). A similar result was obtained with polyA$^+$ RNA isolated from 7 week old plants (data not shown). The very low levels of *GCR1* transcript

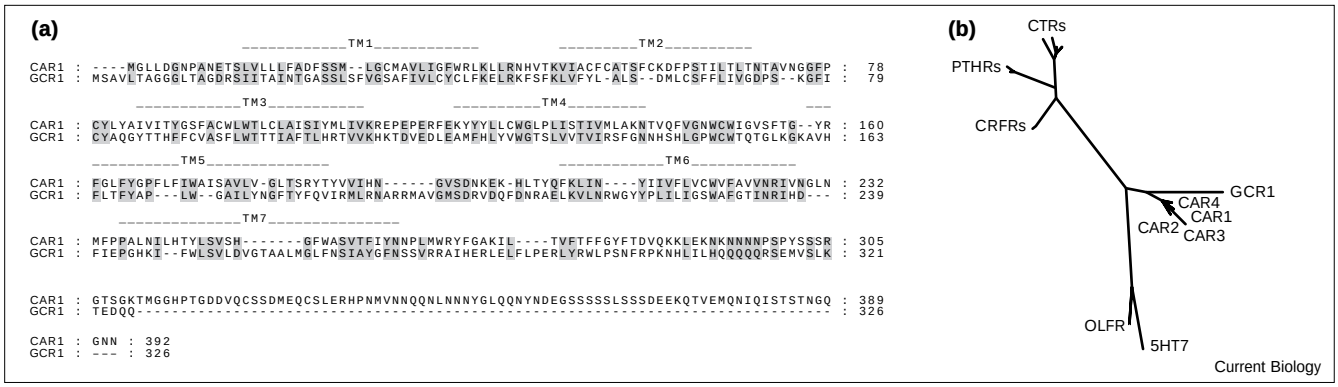
Table 2

Amino-acid sequence similarity and identity values between GCR1 and 7TM receptors.

		Similarity (%)				
		GCR1	CRFR	CTR	CAR1	5HT7
Identity (%)	GCR1	–	46	47	53	45
	CRFR	21	–	57	48	41
	CTR	18	33	–	47	44
	CAR1	23	17	21	–	40
	5HT7	18	20	20	16	–

The deduced amino-acid sequence of GCR1 was compared against *Rattus norvegicus* corticotropin releasing factor receptor 1 (CRFR; GenBank accession number P35353), human calcitonin receptor (CTR; GenBank accession number P30988), a *D. discoideum* cAMP receptor (CAR1; GenBank accession number P13773) and human 5-hydroxytryptamine receptor 7 (5HT7; GenBank accession number P34969). The GAP programme of the GCG package version 8.0 was used, with the default scoring matrix, a gap creation penalty of 3.00 and a gap extension penalty of 0.10.

Figure 3



Comparisons between GCR1 and other 7TM receptors. **(a)** Alignment of the amino-acid sequences of GCR1 and CAR1, a *Dictyostelium* cAMP receptor (GenBank accession number P13773). Shading indicates residues conserved between the sequences. Regions corresponding to membrane-spanning domains are indicated (TM) and numbered. The alignment was generated using the PileUp algorithm of the GCG package (version 8.0) with a GapWeight of 3.0 and GapLengthWeight of 0.1, and is displayed using GeneDoc 1.1.00 [36] with a Dayhoff PAM 250 score table [37]. **(b)** Radial dendrogram showing the relationship of GCR1 with other 7TM receptors. GenBank sequences for the following families of 7TM receptors were compared: family A, M64391 (olfactory receptor, OLFR), P34969 (5-hydroxytryptamine receptor 7, 5HT7); family B, L2332, L2333, X72304, L25438, P35353 and X72305 (corticotrophin releasing factor receptors, CRFRs), L19475, X78936, M77184 and M74445 (parathyroid hormone receptors, PTHRs), L00587, P30988, X69920, M74420, L14617, L14618, L13040, L13041 and X70658 (calcitonin receptors, CTRs); family E, A54813, A46390, P34907, P35352 and P13773 (CARs). No outgroups specified. Sequences of peptides indicated to be most similar to GCR1 were aligned using CLUSTAL V [38], and analysed using the PHYLIP suite of programs [39]. PROTDIST was used to compare sequences by the Dayhoff PAM algorithm [37], NEIGHBOR was used for neighbour-joining cluster analysis [40] and radial phylogenetic trees were drawn using Treeview for Windows. Editing the sequence alignments, by deleting regions of gap introductions, yielded essentially the same tree (data not shown), suggesting that the topology of the tree is robust.

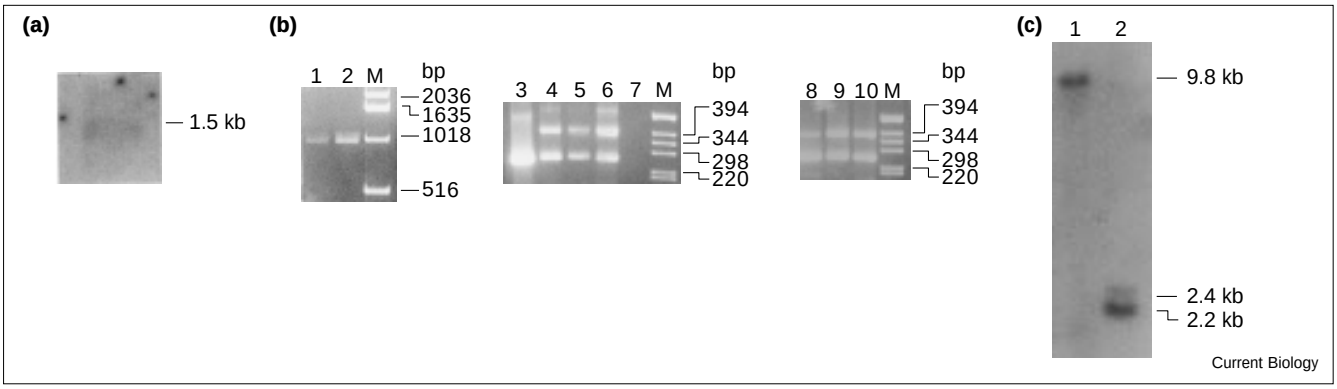
made it impractical to study expression of the gene by Northern hybridisation analysis so we resorted to reverse transcription (RT)–PCR analysis using primers that would distinguish between the transcript and any traces of genomic DNA in the RNA preparations. RT–PCR analysis revealed expression of *GCR1* in 2 week old plants, in stems and leaves of 2 week old and 7 week old plants, and roots of 7 week old plants (Figure 4b). Southern blot analysis indicated that *GCR1* is a single-copy gene (Figure 4c). *GCR1* cDNA was used to screen the *Arabidopsis* CIC library in a yeast artificial chromosome (YAC) vector. Two YAC clones, CIC5G9 and CIC12G3, hybridised to *GCR1*. CIC5G9 is anchored to the RFLP marker m291, which maps to chromosome 5 at 76.4 cM [27].

Functional analysis of *GCR1*

To determine the function of *GCR1*, we transformed *Arabidopsis* with antisense cDNA under the control of the CaMV *35S* promoter. In the second generation (T2), six out of 24 antisense lines produced segregants with a distinct phenotype characterised by reduced cotyledon and leaf expansion and the production of a single flowering stem at the 5–8 leaf stage. We refer to this as a *Dainty* phenotype (Figure 5). The fact that the *Dainty* phenotype appeared in such a high proportion of the antisense lines suggested strongly that it was due to suppression of *GCR1* expression by the antisense construct and implied that *GCR1* might play an important role in plant development. A representative plant, Anti 11, with a single copy of the CaMV *35S* promoter–antisense *GCR1* construct and with the *Dainty* phenotype was studied. PCR analysis confirmed that the antisense *GCR1* co-segregated with the *Dainty* phenotype up to the fifth generation. Using RT–PCR, we determined that native *GCR1* levels in the Anti 11 line were below detectable amounts relative to the vector-only transformants and wild-type plants. Amplimers for actin (*Act2*), which is constitutively expressed, were used as an internal standard for RT and PCR.

Features of the *Dainty* phenotype led us to test the response of Anti 11 plants to cytokinins, plant hormones that influence cell division, apical dominance and flowering [28]. The rationale for this was also supported by the obvious chemical similarities between cytokinins and cAMP, the ligand for the G-protein-coupled receptors to which GCR1 shows the greatest similarity. T3 seeds from an Anti 11 parent with a *Dainty* phenotype were germinated and grown on the cytokinin benzyladenine (BA). Five day old wild-type *Arabidopsis* had roots that were 20 ± 1.5 mm long, and that were reduced by 98% to 0.4 ± 0.1 mm when grown on 4.4 μM BA. *Arabidopsis* carrying the binary vector had roots that were 21.3 ± 1.6 mm long, which were reduced by 96% to 0.8 ± 0.3 mm on BA. The Anti 11 seedlings had roots that were 15.5 ± 1.1 mm

Figure 4

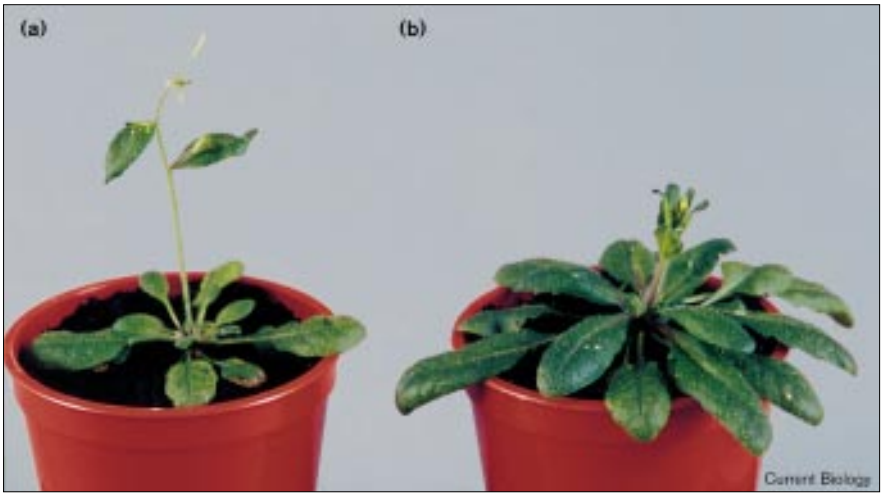


Expression and Southern blot analysis of *GCR1*. (a) Northern blot analysis of polyA$^+$ RNA (9 μg) from *Arabidopsis* seedlings using the *GCR1* cDNA as the probe. (b) RT–PCR analysis of polyA$^+$ RNA isolated from *Arabidopsis* seedlings grown in the light (lane 1) or dark (lane 2) for 4 days; lane 3, positive control using the *GCR1* cDNA clone as the template for PCR; lanes 4–6, RT–PCR analysis of total RNA from whole plant (lane 4), leaves (lane 5) and stems (lane 6) of 2 week old plants; lane 7, no template PCR control, lanes 8–10, RT–PCR analysis of total RNA from stems (lane 8), leaves (lane 9) and roots (lane 10) of 7 week old plants. In lanes 1 and 2, the specific amplification product is 1 kb for this particular amplimer pair; in lanes 4–6 and 8–10, amplimers produce a 265 bp product from cDNA and a 392 bp product from genomic DNA present in the RNA preparations. M, size markers. (c) Southern blot analysis of *Arabidopsis* DNA (10 μg per lane) probed with *GCR1* cDNA. Lane 1, *Eco*RI digest; lane 2, *Hind*III digest.

long, and these were reduced by only 54% to 7.1 ± 0.3 mm on BA. This suggested that antisense *GCR1* had affected the sensitivity of seedlings to BA.

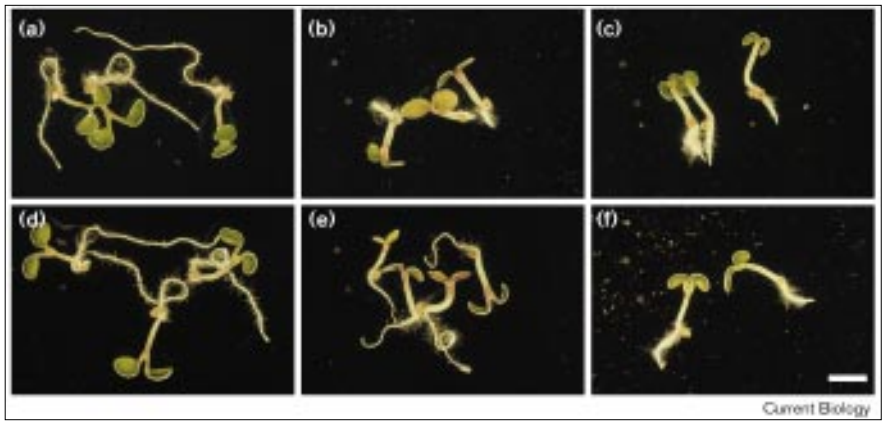
The *Dainty* phenotype and reduced sensitivity to BA were stably inherited into the T4 generation. We therefore tested specificity of the response to BA (Figure 6a–f; Table 3; Figure 7a,b). Inhibition of root growth was significantly lower, over a range of concentrations of BA, in antisense *GCR1*-containing T4 plants compared with the control (Figure 6b,e; Figure 7a). Because cytokinin inhibition of root growth appears to be mediated largely by the induction of ethylene [29], we examined sensitivity to the ethylene-generating compound 1-aminocyclopropane-1-carboxylic acid (ACC). No difference was found between control and antisense seedlings (Figure 6c,f; Table 3). This strongly suggests that the antisense seedlings are specifically affected in their sensitivity to BA. Furthermore, inhibition of root growth by auxin was virtually identical in control and antisense plants (Table 3). Germination suppression by abscisic acid (10 μM), and gibberellin-stimulated (5 μM) hypocotyl elongation were comparable in control and antisense plants (data not shown). Etiolation of control

Figure 5



(a) Mature plant with a *Dainty* phenotype. (b) Vector-only transformant of the same age. Both plants were grown under transgenic greenhouse conditions.

Figure 6



Root growth bioassays. **(a–c)** Control (vector-only transformants) and **(d–f)** Anti 11, T4 seedlings germinated and grown 5 days on medium without hormones as described in the Materials and methods (a,d), or medium supplemented with 6 μM BA (b,e) or 10 μM ACC (c,f). Scale bar = 2 mm.

and antisense seedlings grown for five and seven days in the dark was identical (data not shown), indicating comparable sensitivity to endogenous brassinosteroids [30]. If *GCR1* is playing an important role in cytokinin signal transduction, we would expect reduced sensitivity to cytokinin in other tissues of antisense *GCR1* plants. Cytokinins can overcome etiolation [31]. BA was less effective at reducing hypocotyl elongation in dark-grown antisense *GCR1* seedlings compared with the control (Figure 7b). Thus, *GCR1* influences cytokinin sensitivity in both roots and shoots.

The *Dainty* phenotype is similar to the cytokinin-resistant *Arabidopsis* mutant *cyr1* [32], which maps on chromosome 5, between 76.2 cM and 77.6 cM. We amplified and sequenced *GCR1* from *cyr1* and *CYR1* seedling DNA. The deduced amino-acid sequence of the ORF from *cyr1* and *CYR1* were identical and there were no differences at intron/exon splice sites. Using RT–PCR with actin (*Act2*) as an internal standard, we found that *GCR1* mRNA levels were similar in the homozygous *cyr1* seedlings and wild-type seedlings. *GCR1* does not therefore appear to be involved in the *cyr1* mutation. This is consistent with the *Dainty* and *cyr1* phenotypes being similar but not identical. For example, antisense *GCR1* T3 and T4 plants produce normal flowers that set viable seed, albeit with alterations in time of flowering that are consistent with the known effects of cytokinins. BA-resistant *cyr1* plants produce either no flowers at all, or morphologically defective flowers that do not produce fertile seed [32]. In addition, *Dainty* plants have near wild-type amounts of chlorophyll (data not shown), whereas *cyr1* plants resistant to BA have only 43% of the chlorophyll content of wild-type plants. *CYR1* may therefore encode a closely linked component of cytokinin signalling. The relationship of *CYR1* and *GCR1* to *CKI1*, a histidine kinase homologue implicated in cytokinin signal transduction [33], awaits further investigation. While this manuscript was under review, the cDNA sequence of *GCR1* was reported by Josefsson and Rask [34].

Table 3

The effect of plant hormones on root growth.

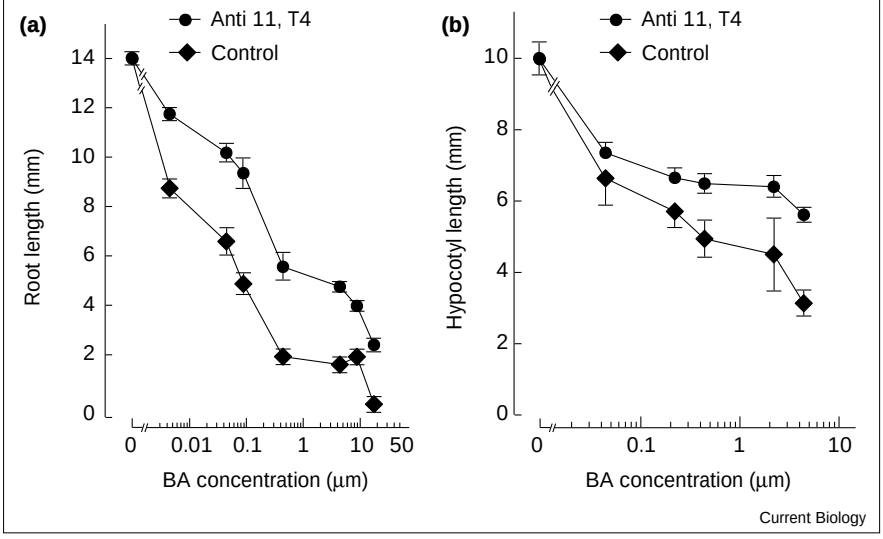
	Root length (mm)		
Hormone	Vector control	Anti 11, T4	*p* value
None	11.9 ± 0.6	11.0 ± 0.5	0.2507*
6 μM BA	1.4 ± 0.3	3.9 ± 0.4	<0.0001†
10 μM IAA	1.7 ± 0.5	1.4 ± 0.2	0.4623*
10 μM ACC	1.8 ± 0.3	1.8 ± 0.3	1.0000*

Root lengths of *Arabidopsis* seedlings were measured 4 days post-germination (mean ± SD, n>20); *p* values were determined by unpaired two-tailed *t* test. *Not significant. †Extremely significant.

Conclusions

We have tested the hypothesis that plant homologues of the superfamily of 7TM receptors exist and, like their counterparts in other organisms, play important roles in responses to endogenous and environmental signals. We have identified the first 7TM receptor homologue in higher plants, *GCR1*, and suggest that other related plant 7TM receptors exist. Homology searches revealed similarities between *GCR1* and the *Dictyostelium* cAMP receptors that led us to explore the possibility that *GCR1* might be involved in the action of a purine signalling molecule. The phenotype of *Arabidopsis* plants expressing antisense *GCR1* supports this theory and suggests that the plant 7TM receptor homologue may be involved in cytokinin signal transduction. This discovery suggests that 7TM receptors are ancient and predate the divergence of plants and animals. It opens new possibilities for understanding signal transduction in plants and presents novel insight into the possible mechanism of cytokinin signalling.

Figure 7



Root and hypocotyl growth bioassays. BA inhibition of root growth and etiolation. Seedlings were germinated and grown in the light (a) or dark (b) for 5 days on medium (see Materials and methods) with or without a range of concentrations of BA. Bars indicate s.e.m.

Materials and methods

Plant material

Arabidopsis thaliana (Colombia) was germinated on tissue culture plates in Murashige and Skoog Basal Medium (Sigma) with 1% agarose and 1.5% sucrose, and maintained in controlled-environment rooms at 22°C with a light/dark regime of 16 h/8 h. Plants for transformation and seed production were grown under transgenic greenhouse conditions with day and night temperatures of 18°C and 15°C (± 2°C), respectively, and supplementary illumination to give a light/dark regime of 16 h/8 h. *Brassica napus* (Fido) was grown from seed under the same greenhouse conditions.

To generate transgenic lines, the flowering parts of 5 week old plants were vacuum infiltrated [35] with *Agrobacterium tumefaciens* (GV3101) carrying the plant binary transformation vector pLARS120. The *Arabidopsis GCR1* cDNA sequence was cloned into the vector in the antisense orientation, under the control of the CaMV *35S* promoter cassette. After 4–6 weeks, seeds were harvested, transformants selected on plates with kanamycin (50 μg/ml) and grown to seed. The selection was repeated to identify single-copy T-DNA insertions and to follow segregation.

Seeds of the mutant *cyr1* were obtained from the *Arabidopsis* Biological Resource Center, Ohio, USA. These were germinated and the homozygous plants distinguished from the heterozygotes and wild type by virtue of their phenotype. DNA for sequencing was prepared 7 days after germination on tissue culture plates. Total RNA was prepared from whole 2 week old seedlings grown under greenhouse conditions.

PCR primers

A series of nested primers were constructed in order to establish the relationship between clone 37H3T7 (dbEST ID 376) and clone VBVIH06 (dbEST ID ATTS2866 and dbEST ID ATTS1113). The two clones proved to overlap. The following primers were used to obtain the *GCR1* genomic and cDNA clones: 5′-GCACGAAACACGAACAGCGGAAATCGT-3′ (forward primer) and 5′-TGTCGTCCAAGAACTCCAAGTTCGTTA-3′ (reverse primer). The following internal gene-specific primers were constructed for the amplification of 5′ and 3′ cDNA ends: 5′-ACAGGTCCGATGAAAGAGTGAAAGCAA-3′ (5′ reverse primer) and 5′-CTGTCATACGTTCTTTTGGTAACAACC-3′ (3′ forward primer). These were paired with the adaptor primers used for the Marathon cDNA library construction (Clontech).

The following primers based on the ORF of *GCR1* cDNA were used to isolate the *B. napus* genomic clone: 5′-TA(T/C)ACXACXCA(T/C)TTTTTTTG(T/C)GT-3′ (forward primer) and 5′-GGT/CTCA/G/TATA/GAAA/GTC(A/G)TG(A/G/T)AT-3′ (reverse primer).

The following primers were designed to produce size-distinguishable products from cDNA and genomic DNA and were used for the RT–PCR: 5′-AA(G/A)GGXTT(C/T)ATTTGTTA(C/T)GCXCA(A/G)GG-3′ (forward primer) and 5′-AATGAACAGCCTTTCCCTTAAGACCAG-3′ (reverse primer)

Preparation of polyA⁺ RNA

Plant material (5 g) was powdered under liquid nitrogen and vortexed with extraction buffer (50 ml; 100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 100 mM NaCl, 0.5% SDS). The aqueous phase was recovered (1,500 *g*, 20 min, 10°C) and added to an equal volume of phenol/chloroform (1:1 v/v). This was shaken (5 min), the phases separated and the process repeated twice more. The aqueous phase was heated (70°C, 10 min) and quenched on ice. 0.5 M LiCl was added followed by 100 mg oligo(dT)–cellulose (Pharmacia), previously equilibrated in binding buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% SDS, 0.5 M LiCl). Gentle mixing ensued (37°C, 30 min) and the oligo(dT)–mRNA was recovered (1,000 *g,* 5 min) and washed twice for 5 min with binding buffer (2 × 10 ml). The RNA was eluted (2 × 10 ml; 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% SDS; 60°C). The eluates were combined and the oligo(dT)–cellulose batch isolation was repeated. The polyA⁺ RNA was precipitated from the final eluate (0.3 M LiCl, 3 vol ethanol, –20°C, 17 h) and recovered (8,000 *g*, 30 min, 2°C). The pellet was resuspended in 300 μl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and clarified in a microfuge (8,000 *g*, 5 min, 4°C). The polyA⁺ RNA was precipitated (0.3 M sodium acetate, 2.5 vol ethanol, –20°C, 60 min) and recovered (8,000 *g*, 10 min, 4°C). The pellet was washed with 70% ethanol.

RACE library construction

A Marathon cDNA RACE library (Clontech) was constructed according to the manufacturer's instructions, and using 1 μg polyA⁺ RNA prepared from 7 day old etiolated *A. thaliana* seedlings. Hot-start PCR was performed using TaqStart Antibody (Clontech) and the Expand Long Template PCR System (Boehringer Mannheim). Six 5′ and four 3′ RACE clones were isolated and sequenced.

RT–PCR

Total RNA was isolated from whole plant, roots, leaves and stems (0.1 g) using the RNeasy Plant Mini Kit protocol (Qiagen). Reverse transcription of total RNA (5 μg) was carried out with Bulk First Strand Reaction Mix (5 μl) from a First Strand cDNA Synthesis Kit (Pharmacia). Aliquots (5 μl) were used in PCR. *Taq* DNA Polymerase (Gibco BRL) was used for the PCR in the Basic Protocol suggested by the manufacturer. Cycling parameters were 94°C, 55°C, 72°C each at 1 min for 30 cycles using a Perkin Elmer 480 DNA Thermal Cycler. Twice-enriched polyA$^+$ RNA was prepared using a Mini Message Maker kit (Ingenius). To determine native *GCR1* mRNA levels in Anti 11 lines, we primed twice-enriched polyA$^+$ RNA for first strand synthesis with the reverse (antisense) primer to *GCR1* and a reverse primer to the constitutively expressed actin (*Act2*) mRNA. The forward and reverse primer pairs were used in subsequent PCR.

Genomic DNA extraction and PCR

Genomic DNA was extracted from leaves (0.1 g) of 4 week old plants using the Easy-DNA Kit (Invitrogen). DNA (100 ng) was used in PCR, 30 cycles in a Perkin Elmer 480 DNA Thermal Cycler, with cycling parameters of 94°C, 55°C, 72°C each at 1 min for *Arabidopsis* DNA and with 2 min at the annealing cycle and 50°C annealing for *B. napus* (Fido) DNA.

Subcloning and sequence analysis

PCR products were subcloned into pCRII (Invitrogen). Plasmids were purified using Plasmid-Tip100 columns (Qiagen). DNA was sequenced using a T7 Sequencing Kit (Pharmacia) and by automated sequencing (J. Hancock, Bristol).

Northern and Southern blots

PolyA$^+$ RNA (9 μg) from 7 day old etiolated seedlings was run on a formaldehyde-denaturing 1% agarose gel and transferred to Hybond N+ (Amersham), hybridised and washed according to manufacturer's instructions. Enzyme-restricted *Arabidopsis* DNA immobilised on Hybond N (Amersham) was hybridised according to the manufacturer's instructions. Probes were generated using *GCR1* cDNA insert and were purified on agarose gels by the Qiaex II procedure (Qiagen). Labelling was with [α-^{32}P]dCTP using an Oligolabelling Kit (Pharmacia). Autoradiography was performed with X-OMAT AR film (Kodak).

Mapping on YAC clones

The CIC library in YAC clones gridded on membranes was a kind gift of C. Dean (John Innes Centre). Filters were probed simultaneously with [α-^{32}P]dCTP-labelled *GCR1* cDNA and [^{35}S]dATP-labelled pYAC4, washed and exposed to two sheets of Kodak Bio Max film to locate the grid of YAC clones and those which hybridised to *GCR1*. DNA was isolated from the two YAC clones that hybridised to *GCR1*, 5G9 and 12G3 [27] and we confirmed by PCR that these clones contained *GCR1*.

Supplementary material

A supplementary figure showing the alignment of the nucleotide sequence of *A. thaliana* GCR1 (GenBank accession number U95142) with the fragment of *B. napus* GCR1 (GenBank accesion number U95144) is published with this paper on the internet.

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